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C3b Receptor in Normal Human Skin

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Receptors for C3b in normal skin were studied. C3b was produced by treating normal human serum with cobra venom factor and by partial digestion of purified C3 with trypsin. Cryostat sections of normal human skin were incubated with C3b, followed by a direct immunofluorescent technique using monospecific goat antihuman C3. The histologic localization of C3b fluorescence was ascertained by fixing cryostat sections with glutaraldehyde and staining with hematoxylin and eosin. The following structures showed staining with anti-C3: (1) endothelial cells in capillaries, larger vessels, and arteries, (2) smooth muscle in arrector pilori muscles and artery walls, and (3) myoepithelial cells in the secretory portion of sweat glands. C3b did not bind to the intercellular substance nor to the basement membrane zone in normal human skin. Normal human sera treated with EDTA, EGTA, and heat (56°C for 30 min) were negative, as was purified C3 by itself, thus indicating that native C3 did not bind to these receptors. Specificity for C3/C3b was shown by blocking with both unconjugated rabbit antihuman C3 and purified C3. The endothelial C3b receptor may have an important role in the localization of immune complexes in cutaneous vasculitis.

Many diseases affecting the skin have been explained as the result of deposition of complement-fixing immune complexes at the dermal-epidermal junction and in the walls of blood vessels. This concept developed from findings by direct immunofluorescence of *in vivo* deposition of complement components and immunoglobulins at these locations [1,2]. Many such patients also have had detectable circulating immune complexes, evidence for consumption of complement, and low levels of complement components or regulatory proteins [3]. The mechanism by which immune complexes deposit in special locations such as the dermal-epidermal junction and walls of cutaneous blood vessels is not known, but the recent observation of Gelfand, Frank, and Green [4], who reported the presence of C3b receptors in glomerular endothelial cells, as well as the loss of these receptor sites in glomeruli of patients with immunologically mediated renal injury [5], suggested to us the possibility that similar receptors also were present in skin. Thus, the present study was undertaken with the objective of determining whether C3b receptors were present in normal human skin.

METHODS

Tissue

Normal human skin was obtained from patients undergoing elective plastic surgery and from volunteers. Multiple cryostat skin sections from 6 different subjects were examined. Biopsy specimens were taken from various sites on the upper and lower extremities, the abdomen, and the chest wall. The specimens were immediately snap-frozen in liquid nitrogen and stored at −70°C until used. Serial cryostat sections, 4 μm thick, were cut fresh each working day. No air drying or fixatives were used.

Preparation of C3 and C3b

From normal human serum: Normal human serum was obtained from whole blood of 3 different healthy donors and was allowed to clot at room temperature for 60 min, after which the serum was separated by centrifugation, aliquoted in 0.1-ml volumes, and stored at −70°C until used. All sera were tested for levels of CH'50, C3, and the presence of smooth muscle or antimitochondrial antibodies; all of these were either within normal limits or negative.

To produce C3b with cobra venom factor (Cordis Laboratories, Miami, FL), a mixture of cobra venom factor (1:2) and normal human serum (1:2) was allowed to react for 30 minutes at 37°C. The pH was then adjusted to between 7.1 and 7.3; EDTA was added to a concentration of 0.01 M, and the material was immediately used on tissue sections.

From purified C3: Lyophilized crystalline trypsin (Worthington Biochemical Company, Freehold, NJ) and soybean trypsin inhibitor (SBTI, Grand Island Biochemical Company), 7,200 units/mg, were reconstituted in Veronal buffer, pH 7.6. Purified C3, 3.3 mg/ml (a gift from Dr. H. J. Müller-Eberhard, Scripps Clinic), was treated with trypsin in a ratio of C3 to trypsin of 100:1 wt/wt for 120 seconds at 23°C. The digestion was stopped by adding a 2-fold weight excess of soybean trypsin inhibitor [6]. Trypsin-prepared C3b was then allowed to react with tissue sections after the addition of 0.01 M EDTA.

Immunofluorescence

Cryostat sections were incubated for 20 min at 25°C with a source of C3 or C3b at a pH between 7.1 and 7.3 [7]. The slides were washed for 10 min in phosphate-buffered saline, pH 7.2, followed by the addition of fluorescein-conjugated anti-C3. After additional washing, the slides were mounted with coverslips in 10% phosphate-buffered saline and glycerin and examined the same day.

Monospecific fluorescein-conjugated goat antihuman C3 (Hyland Laboratories, F/P weight ratio 8.3 μg/mg, F/P molar ratio 3.4, specific antibody concentration 2.2 mg/ml) was used in a dilution of 1:60 (36.6 μg/ml). The specificity was established by Ouchterlony immunodiffusion and by blocking the anti-C3 fluorescence with purified C3. Undiluted rabbit antihuman C3 (Behrings Diagnostics, titer 1.3 mg/ml), purified C3 in a concentration of 0.21 mg/ml, and normal human serum in a 1:128 dilution in 0.01 M EDTA as sources of native C3 were used in the blocking experiments.

The localization of specific immunofluorescence could be directly evaluated by fixing selected cryostat sections in 3% glutaraldehyde for 30 min, followed by staining with hematoxylin and eosin. Photographs of the exact same tissue sites were thus obtained.

Microscope

A Leitz Ortholux II UV-Epiluminator microscope was used. Excitor filter UGI (300 to 400 nm) and barrier filter (430 nm) were used. A 25× fluoride water-immersion objective was used for screening, and a 50× fluoride water-immersion objective was used for photographing. After appropriate modification, the same microscope was used as a light microscope for examining and photographing sections stained with hematoxylin and eosin.

Buffers

The following buffers were used in the study: (1) glucose-gelatin Veronal buffer (gl-gvb^{++} 0.075), pH 7.4, for dilution of normal human serum and cobra venom factor; (2) Veronal buffer, pH 7.6, for the reconstitution of trypsin and soybean trypsin inhibitor; and (3) phosphate-buffered saline, pH 7.2 to 7.3, for the washing of tissues, as one of the controls and to dilute fluorescein-conjugated antibody.

Controls

Phosphate-buffered saline and normal human serum treated with EGTA 0.01 M, EDTA 0.01 M, and heat inactivation (56°C for 30 min) were used as controls.

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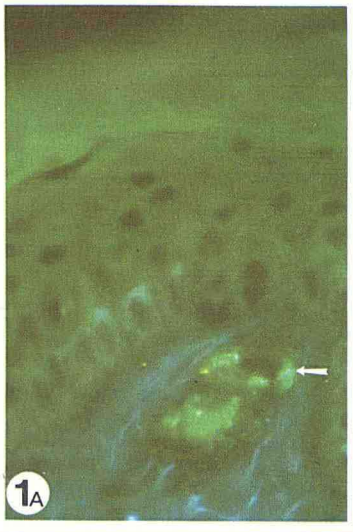
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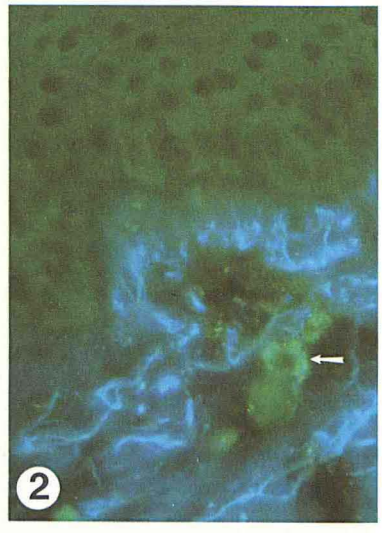
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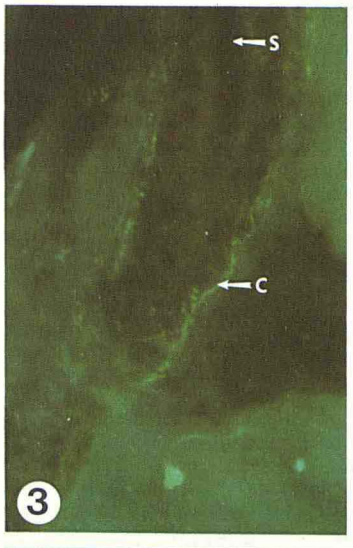
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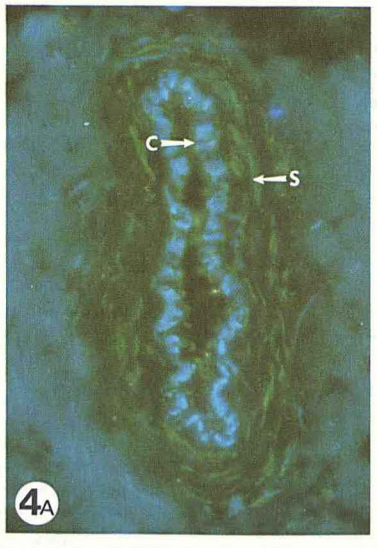
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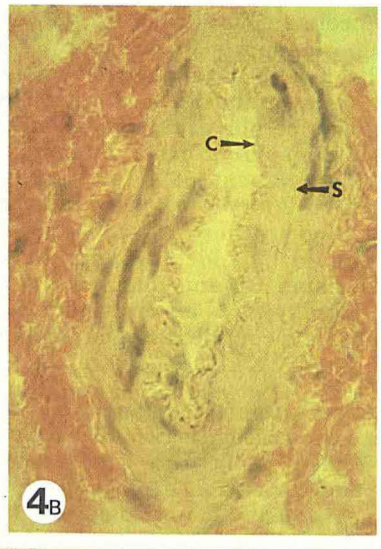
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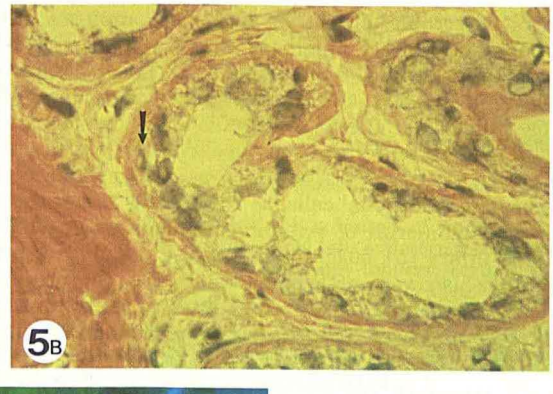
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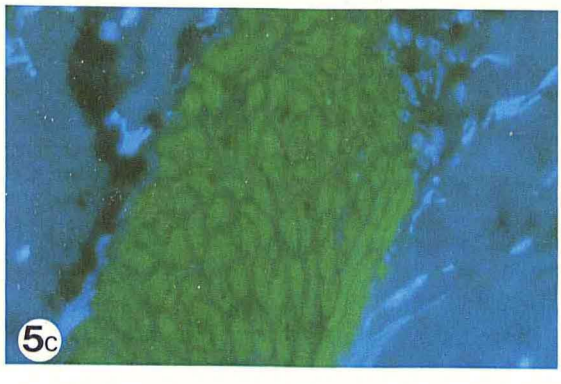
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TABLE I. *Immunofluorescent staining of normal human skin after incubation with serum treated with cobra venom factor*

Structure/cell	Results[a]
Intercellular substance of epidermis	Neg
Dermal-epidermal basement membrane	Neg
Smooth muscle	
Arterioles	++/+++
Arrector pilori muscles	++/+++
Endothelial cells of capillaries, venules, and arterioles	++/+++
Eccrine myoepithelial cells	++/+++

[a] Result of staining with goat FITC anti-C3 (1:60).

Positive Tissue Control

Tissue of normal human kidney was obtained from patients undergoing nephrectomy. Specimens were immediately snap-frozen in liquid nitrogen and stored at −70°C until used. Serial cryostat sections 4-μm thick were cut fresh. No air drying or fixatives were used. Cryostat sections of normal human skin were also used, prepared as described above. Cryostat sections of both tissues were incubated for 20 min at 25°C with a source of cobra venom factor-treated normal human serum (see above, preparation of C3), as well as a source of cobra venom factor-treated normal human serum spontaneously decayed for 24 hr at 37°C. Both sera were used at pH between 7.0 and 7.3. The slides were washed for 10 min in phosphate-buffered saline, pH 7.2, followed by the addition of fluorescein-conjugated anti-C3 (Hyland Laboratories) and fluorescein-conjugated anti-IgG in a dilution of 1:20 (Behring Diagnostic Laboratories, F/P molar ratio 2, specific antibody concentrations 65 μg/ml). After additional washing, the slides were mounted with coverslips in 10% phosphate-buffered saline and glycerin and examined the same day.

RESULTS

In preliminary experiments, we used inulin particles coated with C3b in order to detect C3b receptors but had difficulty in defining localization precisely. Normal human serum treated with cobra venom, followed by EDTA, proved to be a much better reagent. EDTA was added because, in its absence, positive staining by serum not treated with cobra venom factor sometimes occurred, owing to the conversion of C3 to C3b, probably by enzymes present in tissues [8]. Although staining of the dermal-epidermal junction was never seen, C3b receptors could be identified in arteriolar smooth muscle, the arrector pilori muscles of hair follicles, and the endothelial cells in the capillaries located in the dermis, papillary dermis, and around the sweat glands (Table I). Staining was also seen in the endothelial cells lining the lumina of venules and arterioles. In the secretory portion of eccrine sweat glands, the myoepithelial cells also showed positive fluorescence.

Endothelial cells were brightly stained by serum treated with cobra venom factor but not by EDTA serum (Fig 1 *A*). The endothelial cell identification was demonstrated by comparison of the section stained using anti-C3 with the same one stained using hematoxylin and eosin (Fig 1 *B*). That the staining of capillary endothelial cells actually represented binding of C3b was demonstrated by the absence of binding of purified C3 but binding to the endothelial cells of the same material converted to C3b by trypsin. Positive staining was seen after treatment with trypsin-treated C3 but not with native C3 (Fig 2). Similar fluorescence was seen in the endothelial cells that lined the capillaries surrounding sweat ducts, small venules, and arterioles, and smooth muscle in the vessel wall (Fig 3 and 4). The immunofluorescent pattern and histologic structure of the myoepithelial cells of the secretory portion of eccrine sweat glands, as well as the immunofluorescence of the arrector pilorus muscle of a hair follicle, are shown in Fig 5.

TABLE II. *Immunofluorescent staining of normal human skin with purified C3 before and after treatment with trypsin*

Structure/cell	Result[a]	
	Incubation with purified C3	Incubation with trypsinized C3
Intercellular substance of epidermis	Neg	Neg
Dermal-epidermal basement membrane	Neg	Neg
Smooth muscle		
Arterioles	Neg	+++
Arrector pilori muscles	Neg	++++
Endothelial cells of capillaries, venules, and arterioles	Neg	++/+++
Eccrine myoepithelial cells	Neg	++/+++

[a] Result of staining with goat FITC anti-C3 (1:60).

TABLE III. *Blocking experiments to demonstrate specificity of fluorescein-conjugated goat antihuman C3*

First stage	Second stage	Third stage	Result
Normal human skin incubated with:			
Cobra venom-treated serum	Rabbit anti-C3 (unconjugated)	Goat anti-C3 FITC (1:120)	Neg
Cobra venom-treated serum	Saline	Goat anti-C3 FITC (1:120)	Pos
Purified C3 treated with trypsin	Rabbit anti-C3 (unconjugated)	Goat anti-C3 FITC (1:120)	Neg
Purified C3 treated with trypsin	Saline	Goat anti-C3 FITC (1:120)	Pos
Normal human skin incubated with:	Mixture of goat anti-C3 FITC (1:120) and:		
Cobra venom-treated serum	Normal human serum 1:128		Neg
Cobra venom-treated serum	Purified C3 (0.21 mg/ml)		Neg
Cobra venom-treated serum	Saline		Pos

The fluorescent pattern of the endothelial cells of blood vessels consisted of linear and wavy fluorescent streaks that appeared to represent surface staining, whereas the myoepithelial cells of sweat glands and the smooth muscle cells of blood vessels and hair follicles showed a much more homogeneous pattern, consistent with staining of intracellular structures. However, it was not possible from immunofluorescent histology to localize staining precisely. The results with purified C3 before and after treatment with trypsin duplicated those obtained with human serum (Table II).

Unconjugated rabbit anti-C3 obtained from Behring Diagnostic Laboratories blocked staining by fluoresceinated goat

FIG 1. Localization of receptors for C3b in capillary endothelium. *A,* Section of skin incubated with cobra venom-treated human serum followed by FITC anti-C3. *Arrow* indicates endothelial staining (×500). *B,* Same section stained with hematoxylin and eosin. *Arrow* indicates endothelium (×500).

FIG 2. Incubation of skin section with trypsin-treated purified C3, followed by FITC anti-C3. *Arrow* indicates endothelial staining (×500).

FIG 3. C3b fluorescence of endothelial cells of capillaries surrounding a sweat duct. *S* indicates lumen of duct; *C* indicates capillaries.

FIG 4. Presence of receptors for C3b in arterial smooth muscle. *A,* Section incubated with normal human serum treated with cobra venom, followed by FITC anti-C3. *S* indicates staining of smooth muscle; *C* indicates staining of endothelium. *B,* Same section stained with hematoxylin and eosin.

FIG 5. Presence of receptor for C3b in myoepithelial cells of eccrine sweat glands and arrector pilorus muscle. *A,* Section incubated with normal human serum treated with cobra venom, followed by FITC anti-C3. *Arrow* indicates myoepithelial cell fluorescence. *B,* Same section stained with hematoxylin and eosin. *Arrow* indicates myoepithelial cell. *C,* C3b binding to arrector pilori muscles. Section incubated with normal human serum treated with cobra venom, followed by FITC anti-C3.

TABLE IV. *Control studies of immunofluorescent staining of normal human skin by anti-C3 to establish tissue binding of C3b but not C3*

Normal human skin incubated with:	Goat anti-C3 FITC (1:60)
Normal human serum 1:4 (0.01 M EDTA)	Neg
Normal human serum 1:4 (0.01 M EGTA)	Neg
Normal human serum (56°C 30 min)	Neg
Phosphate-buffered saline	Neg
Normal human serum 1:4 treated with cobra venom factor	+++

TABLE V. *Positive tissue control study: Immunofluorescent staining of normal human kidney glomeruli and normal human skin with fresh and decayed cobra venom factor-treated normal human sera*

First step	Second step	Kidney glomeruli	Human skin
Cobra venom-treated serum	Goat anti-C3 FITC (1:60)	Positive	Positive
Cobra venom-treated serum	Goat anti-IgG FITC (1:20)	Negative	Negative
Decayed cobra venom-treated serum	Goat anti-C3 FITC (1:60)	Negative	Negative
Normal human sera, 0.01 M EDTA	Goat anti-C3 FITC (1:60)	Negative	Negative
Phosphate-buffered saline	Goat anti-C3 FITC (1:60)	Negative	Negative

anti-C3 (Hyland Laboratories) of both C3b in serum and C3b obtained by trypsinization of C3 (Table III). Purified C3 in a concentration of 0.21 mg/ml and normal human serum in EDTA diluted 1:128 both obliterated the fluorescence produced by 1:120 fluoresceinated goat anti-C3. All of these results established the specificity of the direct immunofluorescent technique utilized in the present study.

Endothelial cells, smooth muscle cells, or myoepithelial cells did not fluoresce if the first stage consisted of normal serum treated either with EDTA or with EGTA or by heating at 56°C for 30 min (Table IV). The goat anti-C3 conjugate by itself did not produce any staining.

Receptors for C3b are known to be present on human kidney glomeruli; thus, this tissue is suitable to use as a positive control to demonstrate specificity of our immunofluorescence technique. Incubation of fresh cobra venom-treated normal human serum on cryostat sections of kidney glomeruli showed positive staining demonstrated by fluorescein-conjugated anti-C3. Fluorescein-conjugated anti-IgG was also used to demonstrate that the staining is not due to an antibody. No positive staining could be demonstrated by fluorescein-conjugated anti-IgG. Cryostat sections of kidney glomeruli incubated with decayed cobra venom factor-treated normal human serum showed no binding to the glomeruli. The same experiments performed on cryostat sections of normal human skin gave results identical to those of the kidney glomeruli. The results of the positive tissue control are listed in Table V.

Our results, blocking experiments, controls, and positive tissue controls indicate that a receptor for C3 is present in normal human skin, and most likely the specific staining demonstrated in normal human skin is due to the C3b subcomponent.

DISCUSSION

Our study demonstrates the presence of C3b receptors of endothelial cells, smooth muscle cells, and myoepithelial cells of eccrine sweat glands. In an *in vitro* technique, C3b (produced by cobra venom factor treatment of normal human serum and trypsinization of purified C3) showed binding to cryostat sections of normal human skin.

Previously, C3b receptors have been found on the surface of a large number of circulating cells as well as to some extent on fixed cells. In the former category, such cells as B-lymphocytes, erythrocytes, platelets [9], and neutrophilic granulocytes [10] have been shown to possess C3b receptors on their surfaces; and among tissue cells, the Langerhans cells [11] and the rat mast cells [12] have similar receptors.

The C3b receptor on these cells is involved in various functions. Phagocytosis by polymorphonuclear leukocytes and macrophages is enhanced by C3b receptors, and the receptor is also involved in the neutralization of viruses [13,14]. C3b present in immune complexes composed of T-lymphocyte dependent antigens and IgM antibody is believed to facilitate the binding of such complexes to B-lymphocytes and monocytes via the membrane receptor, thereby enhancing antibody production [15]. Miller and Nussenzweig [16] have postulated that complement may regulate the interaction between immune complexes and cell membranes. They found that initially complement components incorporated into immune complexes increased the affinity of the complexes for cell membranes. Subsequently, the immune complexes were converted to a nonbinding state by a mechanism involving the alternative complement pathway. Cell-bound complexes could be released from the cell membrane by this second mechanism.

Investigators who have studied the localization of immune complexes in tissues have postulated several possible ways by which they could be fixed. Among the factors that affect deposition of immune complexes in blood vessels are high molecular weight and release of histamine by basophils and platelets. Histamine increases the permeability of blood vessel walls, resulting in trapping of immune complexes [17,18]. In addition, size of blood vessels, turbulence, local damage to vessel walls, hypertension, and solubility of immune complexes also have a role in this process. Gelfand, Frank, and Green [4] reported the presence of C3b receptors on human glomerular endothelial cells and suggested that these receptors are responsible for the localization of circulating immune complexes in the kidney. Evidence supporting this concept was their subsequent finding that renal diseases characterized by the presence of circulating immune complexes and in vivo deposition of C3 were associated with a loss of receptor sites on glomerular endothelium [5]. However, not all immune complexes found in tissues are derived from the circulation. Couser et al [19] and Couser et al [20] demonstrated that subepithelial tubular immune deposits can be produced in the isolated rat kidney by perfusion with specific antibody to rat proximal tubular epithelial cell brush border antigen in the absence of circulating immune complexes. Thus, deposition of immune complexes can result from *in situ* formation rather than from circulation deposition. Electrophysical characteristics of the glomerular capillary wall may influence complex localization in the glomerular basement membrane.

Our study did not demonstrate a C3 receptor present at the dermal-epidermal junction. Immune deposits at this location are frequently seen in diseases with circulating immune complexes such as systemic lupus erythematosus (SLE). In SLE, localization of such immune complexes may be due to binding of DNA in the complexes by collagen receptors, with the subsequent deposition of antibody or trapping of immune complex mechanically [21].

In the interim, before our results were published, a study by Schreiber and Penny [22] noted negative results in human skin. We believe that the authors used a technique that was too insensitive to be able to detect potential C3b receptors in human skin, namely fluorescein-labeled *S. typhi* coated with C3b, as well as autopsy specimens. Our technique is far more sensitive and useful for human skin, in which the density of receptors most likely is less than that of kidney glomeruli. We also made a special effort to obtain fresh tissue for our experiments. The difference in technique may explain our positive findings.

Our finding of a C3b receptor of the endothelial cells in capillaries and in arterioles and venules may be of importance in the localization of immune complexes at these sites and may have an important role in the immunopathogenesis of the cutaneous vasculitides in which circulating immune complexes

have a major role. Evidence for such a role has been demonstrated in the animal models of the Arthus reaction and serum sickness [23]. Immunoglobulins and complement components also have been demonstrated by direct immunofluorescence studies in the cutaneous vessels in both early and late lesions of vasculitis [24]. The C3b endothelial receptor may provide the anchor by which immune complexes initially localize to these tissues, followed by subsequent release of C3a and C5a, with attraction of granulocytes and liberation of histamine leading to vessel injury. C3b receptors on endothelial cells probably are widespread throughout the body.

The finding that C3b also binds to smooth muscle in the arrector pilori muscles, arterioles, and myoepithelial cells was unexpected. We have not yet established whether C3b is bound to surface or intracellular sites in smooth muscle and myoepithelial cells of eccrine sweat glands. Binding to these cells may be related to their content of myofibrils. The histologic pattern of C3b binding to these structures is suggestive of an intracellular localization (Fig 4 and 5).

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